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ANTHER DEVELOPMENT IN THE STAMINATE FLOWER OF THE LODGEPOLE PINE DWARF MISTLETOE, *ARCEUTHOBIUM AMERICANUM*

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ANTHER DEVELOPMENT IN THE STAMINATE FLOWER OF THE LODGEPOLE PINE DWARF MISTLETOE, *ARCEUTHOBIUM AMERICANUM*

by

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Dated this 1st day of May, 2013, in Kamloops, British Columbia, Canada

ABSTRACT

Dwarf mistletoes (genus *Arceuthobium*) are parasitic angiosperms that infect commercially valuable conifer trees across North America, resulting in millions of dollars of damage annually. The purpose of this study was to examine the developmental processes taking place during anther development in the dwarf mistletoe species, *Arceuthobium americanum,* and to describe the eventual three-dimensional relationship between the anther and the columella (a region of sterile tissue within the anther). Angiosperms typically have anther walls comprised of several layers. However, the composition of the anther wall layers and aspects of layer development varies from species to species. These processes have only been superficially examined in the *Arceuthobium* genus. In order to obtain insight into anther wall development in *A. americanum*, thin sections were cut, mounted and stained on slides, and viewed with a light microscope. The data indicate that *A. americanum* have three distinct anther wall layers including 1) the epidermis, 2) the middle layer, and 3) the tapetum. Most angiosperms have a sub-epidermal layer called the endothecium, but this work shows that *A. americanum* possesses an "exothecium" similar to that of gymnosperms. The eventual three-dimensional orientation of the anther with reference to the columella was described using a combination of light and dissecting microscopy. Previously, it was thought that the columella spanned the length of the anther to such an extent that the loculus (anther chamber) was bisected; however, this study reveals that the columella does not span the length of the anther in either direction, even though it does originate centrally.

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INTRODUCTION

Dwarf Mistletoes (genus *Arceuthobium*)

Dwarf mistletoes (genus *Arceuthobium*, Santalaceae) are destructive parasites that are of both economic and ecological importance to Canadian forests (Hawksworth and Wiens 1996). The genus *Arceuthobium* was initially ascribed to the family Viscaceae (Davis 1966), but that family has since lost its status, and its members have been reassigned to the parasitic family Santalaceae (APG II 2003). The parasitic condition in plants has arisen approximately eleven times, encompassing around 4,000 species from 265 genera and twenty-two families, which roughly works out to represent 1% of all angiosperms (Nickrent et al. 1998). The vast majority of parasitic angiosperms do not elicit detectable negative effects upon their host; however, *Arceuthobium* spp. do.

Roughly forty-four different species of dwarf mistletoes exist, dispersed as parasites on members of either Pinaceae or Cupressaceae in Canada and the United States, and almost all species of coniferous trees have an associated species-specific *Arceuthobium* infection (Hawksworth and Wiens 1996; Jerome and Ford 2002). This type of species-specific, parasite-host relationship is common to all domains of life. In the genus *Arceuthobium*, for example, *A. pusillum* mainly infects *Picea glauca* (white spruce) and *Picea mariana* (black spruce), while *A. americanum* primarily infects *Pinus contorta* (lodgepole pine) and *Pinus banksiana* (jack pine) (Hawksworth and Wiens 1996).

Arceuthobium americanum, however, is capable of infecting up to fourteen different conifers from the Pinaceae family, which is the most extensive range of infection of any North American dwarf mistletoe species (Jerome and Ford 2002; Hawksworth

and Wiens 1996). This is problematic to foresters, as conifers infected with dwarf mistletoes show an overall reduction in growth (Chhikara and Ross Friedman 2008; Hawksworth and Wiens 1996), reproductive fitness, and wood quality, which equates to extensive product loss and, by extension, monetary losses to the lumber industry (Hawksworth and Wiens 1996). In 1982, for example, it was estimated that dwarf mistletoe infections in Canada and the Western United States were responsible for monetary losses upwards of $2.6 billion dollars, based on a steady rate of infection (Drummond 1982).

Arceuthobium americanum is a small, dioecious, yellowish to olive green herb, consisting of aerial shoots with whorled branching (Hawksworth and Wiens 1996), connected to a network of cortical strands and radial sinkers that penetrate the coniferous host (Calvin and Wilson 1996). The mistletoe invades the aboveground parts of the host and is able to penetrate through the thin, chlorophyllous bark of conifer branches, some as old as 60 years (Hawksworth 1954). Once infection (seedling establishment) has occurred, shoots can take up to five years to emerge from the bark. This shoot development is generally climate-dependent: *Arceuthobium* infections in British Columbia produce shoots in the second and third years after infection (Smith 1971), whereas infections in Alaska take three to six years to develop shoots (Shaw and Loopstra 1991). The host usually shows signs of swelling at the infection point (Hawksworth 1954), which can precede shoot development by more than a year. Aerial shoot tips with developing inflorescences consist of a single terminal floral bud (that is, the bud at the apex of the stem) superior to two lateral buds (the floral buds found on the side of the stem), which are opposite in arrangement (Figure 1i).

Figure 1i. The terminal portion of the staminate male) shoot showing a lone mature terminal bud and inferior lateral flower buds in opposite arrangement (image adapted from Cohen 1968).

Staminate (male) reproductive structures (flowers) consist of three sepals and no petals: some older studies refer to the sepals as 'tepals' because it was previously unclear as to whether these floral parts were developmentally sepals (i.e., comprising the outermost whorl) or petals developing from an inner region (Cohen 1968). Each sepal acts as a holding pad for a single sessile anther. Central to the three sepals is a tri-lobed pad-like elevation of sterile tissue known as the central cushion, which excretes extremely sugary nectar (Penfield et al. 1976). Dehiscence of pollen from the anther is via a transverse slit in the anther's epidermis, the anther's outermost layer (Gilbert and Punter 1990). Each male flower produces about 11,000 pollen grains per its three anthers (Penfield et al. 1976). In response to temperature and humidity changes, each anther is capable of reversibly opening and closing (Gilbert and Punter 1990).

Overview of Anther Development in Angiosperms and *Arceuthobium* spp.

In typical angiosperms (Maheshwari 1950), a very young anther consists of actively dividing meristematic cells, which are surrounded by a single layer of epidermal tissue (Figure 2i). Development of microsporangia (anther or pollen sacs) begins

with the differentiation of archesporial cells in the region below the epidermis (i.e., the hypodermal region, Figure 2iA). The archesporial cells divide by periclinal division (divisions parallel to the surface, Figure 2iB) to give a subepidermal primary sporogenous layer and a primary parietal layer. The former undergoes periclinal and anticlinal divisions (divisions perpendicular to the surface) to form a mass of sporogenous tissue within the microsporangium and tapetum. Once divisions have ceased, the cells are referred to as microsporocytes. These microsporocytes undergo meiosis and mitosis to form microspores (immature pollen grains) and mature pollen grains, respectively.

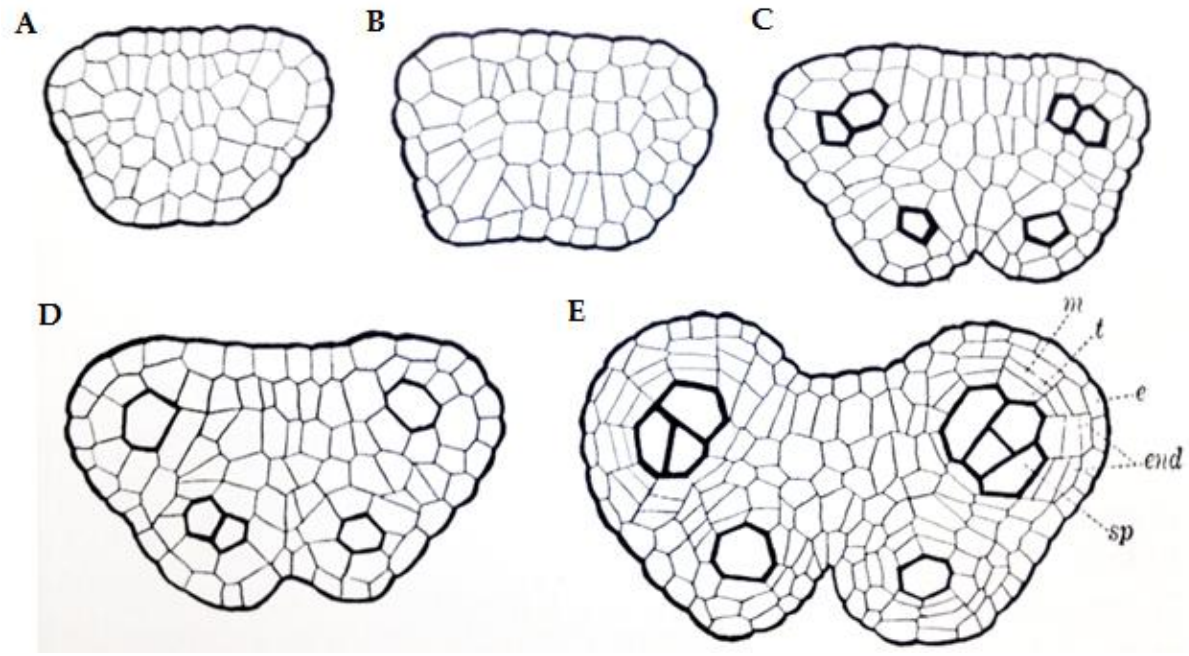


Figure 2i. Development of a typical tetrasporangiate angiosperm anther (i.e., having four microsporangia). **A.** Very early in development anthers present with a mass of homogeneous meristematic cells surrounded by the epidermis. **B.** After a few periclinal divisions. **C-E.** Continued development leading to the origin of the sporogenous tissue. Legend: sp, sporogenous tissue; t, tapetum; m, middle layer; end, endothecium; e, epidermis (image adapted from Maheshwari 1950).

Meanwhile, as the sporogenous cells are developing, cells of the primary parietal layer divide periclinally (along with some anticlinal divisions) to form cell layers found external to the microsporangium, which together comprise the anther wall (Maheshwari 1950; Davis 1966). The layers that make up the four main constituents of the mature angiosperm anther wall are (from outermost to innermost): the epidermis, the endothecium (a layer with fibrous thickenings), the middle layer, and the tapetum (Maheshwari 1950; Davis 1966; Dundas et al. 1982; Bedinger 1992). Based on mitotic division patterns and the eventual differentiation of a secondary parietal layer (if such a layer is develops), species of angiosperms can be categorized into one of four patterns of anther wall development (Figure 3i) (Davis 1966). The four types of anther wall development are basic, dicotyledonous, monocotyledonous, and reduced.

Typically, angiosperms are tetrasporangiate (as in Figure 2i), becoming bisporangiate at the time of dehiscence, owing to the breakdown of the cells in the septum partitioning the two microsporangia of the same lobe. Dowding (1931) determined that *Arceuthobium* spp., however, are actually uni- or monosporangiate (i.e., developing only one microsporangium), although it remains unclear whether or not the anther is monosporangiate from its inception (Dowding 1931) or is initially bisporangiate and undergoes septum breakdown (Mauseth 1988).

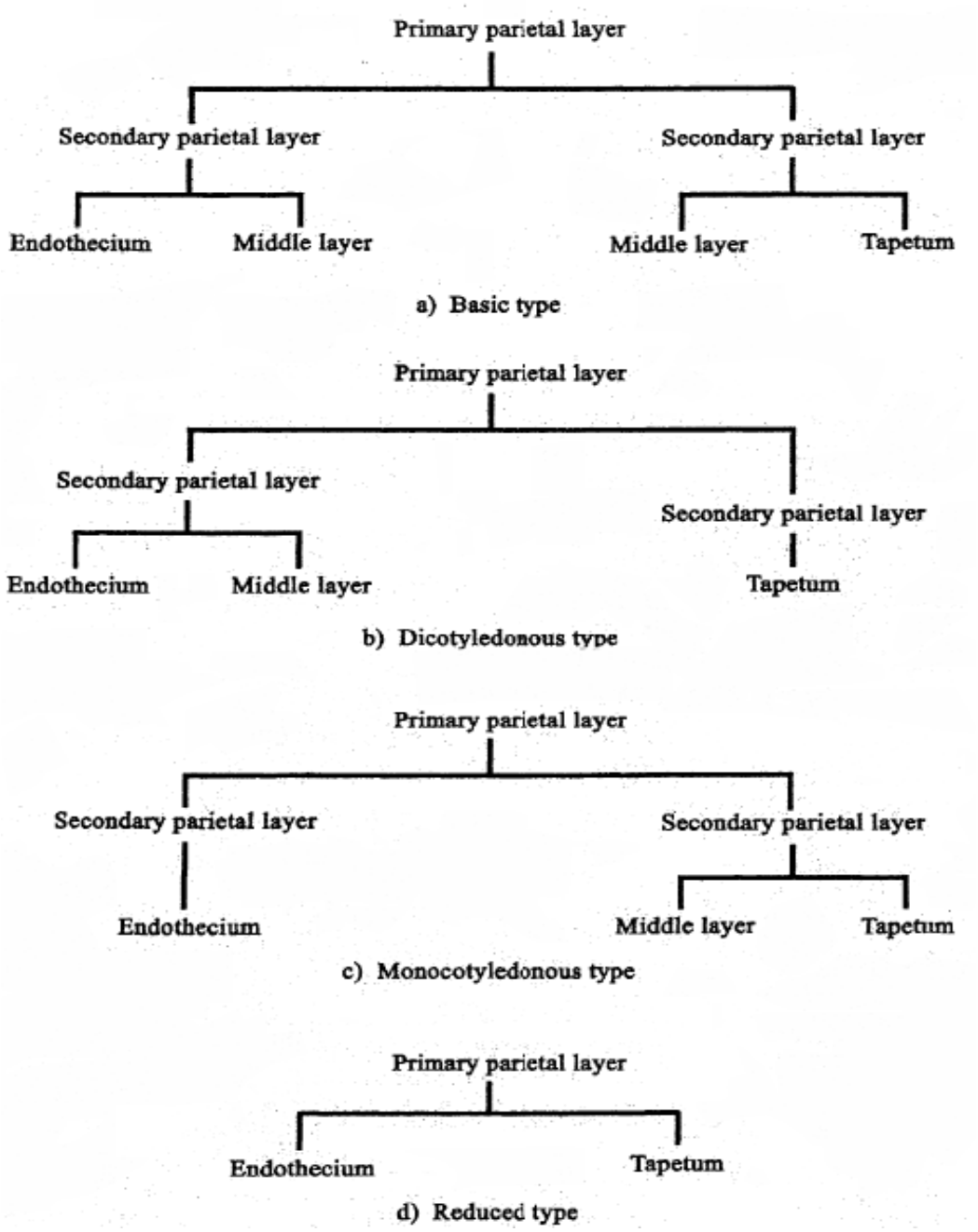


Figure 3i. The four patterns of anther wall development in angiosperms (Davis 1966).

Most angiosperms develop an endothecial layer, which is commonly a single layer of cells (Maheshwari 1950; Davis 1966; Vasil 1967; Dundas et al. 1982). In some angiosperms, a bi- or even multilayered endothecium can arise via periclinal

divisions; however, a layered endothecium is only likely to occur in more primitive angiosperm families (Tobe 1989). The cells that comprise a typical endothecium are radially elongated, and fibrous bands of callose, which arise from the inner tangential walls, run up and out, coming to an end near the outer wall of each cell (Maheshwari 1950; Vasil 1967). These fibrous bands provide structure and support to the anther and also function in anther dehiscence (Goldberg et al. 1993). Some species do not have fibrous thickenings, and dehiscence takes place when cells at the apex of the anther undergo programmed cell death (Maheshwari 1950).

In contrast to other angiosperms, neither *A. oxycedri* nor *A. americanum* are thought to have an endothecium. Rather, the epidermis of these species' anthers develops fibrous wall thickenings, thus making the epidermis an 'exothecium' (Dowding 1931). The term 'exothecium' has been used to describe the epidermis alone, both the epidermis and the endothecium, or the outermost layer of the anther wall (Dowding 1931; Skene 1948; Bhatnagar and Moitra 1996). Although the term exothecium has been used in a manner that suggests a broad range of meanings, generally, an exothecium seems to be an all-encompassing term that describes the active layer with fibrous thickenings in the absence of an endothecium, whereas the endothecium is defined by its sub-epidermal location in addition to the presence of fibrous thickenings (Skene 1948; Shukla et al. 1998).

Typically, the angiosperm middle layer only persists for a short time, as these cells are unable to divide anticlinally and are eventually crushed (Davis 1966; Anjaneyulu and Lakshminarayana 1989). Middle layers that do persist usually do so because they have developed fibrous wall thickenings similar to the endothecial layer (Vasil 1966; Hardy et al. 2000). The number of middle layers ranges from zero to seven, but commonly there are one to three layers (Maheshwari 1950). These middle layers

give structure and support to the anther and, if retained, can be involved in dehiscence (Goldberg et al. 1993; Hardy et al 2000).

The tapetal layer of angiosperms is highly specialized and of considerable developmental significance (Maheshwari 1950; Davis 1966; Scott et al. 2004). The tapetum is the innermost anther wall layer, which supplies the nutrients and necessary substances, such as macromolecules, e.g., lipids (Piffanelli et al. 1998), to the reproductive cells in the anther chamber or loculus (Maheshwari 1950; Bedinger 1992; Goldberg et al. 1993). Previously, tapetal cells were thought to originate from primary parietal cells (Maheshwari 1950), but some authors have reported origination from more than one tissue type, such as the secondary parietal layer or from connective tissue (Davis 1966; Anjaneyulu and Lakshminarayana 1989). In most angiosperms, the tapetum is a single layer of cells with some known cases of bi- or multilayer conditions (Davis 1966; Furness & Rudall 2001). Most often the production of the tapetum (i.e., mitosis) is completed before the development of the microsporocytes, but occasionally the tapetal cells undergo mitotic divisions during the meiotic divisions of the encased reproductive cells.

Two main types of tapeta have been described, the secretory and the amoeboid (Maheshwari 1950; Davis 1966). These two tapeta differ in their methods of microspore nutrition; that is, all substances produced by the secretory tapetum reach the developing pollen grains via the locular fluid (a medium infiltrating the space between each developing pollen grain). In contrast, the locular fluid is greatly reduced in volume or completely absent in amoeboid tapeta. Instead, the amoeboid tapetal cell wall breaks down, similar to the secretory tapetum, but their protoplasts fuse and a multinucleate plasmodium protrudes into the loculus (Furness and Rudall 2001; Pacini et al 1985). Surrounding this protruding structure is the tapetal

membrane (Furness and Rudall 2001) or a culture sac (Pacini et al. 1985). In contrast to the secretory type, the amoeboid type is a form of direct nutrition, as the amoeboid tapetum directly surrounds the developing microsporocytes.

The most common tapetum is the secretory tapetum (Maheshwari 1950; Davis 1966; Furness and Rudall 2001), which can also be referred to as glandular, parietal (Pacini et al. 1985), or cellular (Greyson 1994). The amoeboid tapetum has also been referred to as (peri) plamodial (Greyson 1994; Furness and Rudall 2001). A few angiosperm families present with intermediate forms of tapeta, but these have not been previously well characterized (Pacini et al. 1985); however, Furness and Rudall (2001) introduced the term 'invasive nonsynsytial' as a possible description for the intermediate type tapetum. The cells of a secretory tapetum act as nurse cells by providing microsporocytes with nutrients such as polysaccharides and proteins as well as saturated and unsaturated lipids (Maheshwari 1950; Davis 1966; Pacini et al. 1985; Clement et al. 1998; Piffanelli et al. 1998). These nutrients must pass through the locular fluid, therefore indicating an indirect form of nutrition. Tapetal cells are known to degenerate in order to free certain secretion products such as lipidic components, which contribute to the formation of the pollen coat (Pacini 1990; Piffanelli et al. 1998). They do so in a typical pattern, albeit via different mechanisms and at different times (Pacini et al. 1985; Pacini and Franchi 1991).

Regardless of tapetal type, it is generally understood that the relationship between the tapetum and the developing sporogenous tissue is of utmost importance (Davis 1966; Pacini et al. 1985; Bedinger 1992), which has been confirmed by recent molecular genetic knockout experiments in *Arabidopsis*, where a number of transcription factors were knocked out (Zhu et al. 2011). Bedinger (1992) also noted

that morphological irregularities (e.g., fewer or absent mitochondria) in tapetal cells have been observed in male sterile species prior to pollen abortion.

The Columella: A Unique Feature of the *Arceuthobium* Anther

Thoday and Johnson (1930) and Dowding (1931) reported the presence of a central sterile column of tissue surrounded by sporogenous tissue in *A. pusillum* and *A. americanum*, respectively. Dowding (1931) called this sterile column a 'columella'. The columella has been considered a unique feature to the genus *Arceuthobium* (Maheshwari 1950). In other angiosperms (e.g., *Ricinus communis*) a central septum has been reported, but the term 'columella' and the specific tissue it represents seems to be reserved for the genus *Arceuthobium*. The only clear anatomical and/or morphological difference between the septum and columella seems to be that the cells of the septum degenerate prior to dehiscence and also that the septum fully partitions the loculus. Dowding (1931), however, suggests that the protruding edges of the columella are evolutionary remnants of a septum that might have partitioned an bisporangiate anther of some ancestor. The three-dimensional positioning of the columella has been the subject of much disagreement for many years, beginning in 1931 when Dowding described the columella as exhibiting a considerable amount of orientational variation. The description of the relationship between the columella in the mature anther has yet to be discerned with modern microscopy.

Pollen Development in Angiosperms and *Arceuthobium* spp.

Meiosis of microsporocytes in the anthers gives rise to haploid microspores or immature pollen grains (Maheshwari 1950). A time after microsporocyte meiosis, an asymmetric, mitotic division results in two nuclei within the microspore, referred to as the generative and vegetative nuclei; at this point the pollen grain is said to be

mature. Generalized illustrations by Bedinger (1992) depicting pollen development in maize suggest that these two nuclei migrate from a central location to a peripheral one. A plasma membrane with limited cell wall material typically circumscribes the generative nucleus as its own cell, while the vegetative nucleus remains in the confines of the microspore (and can thus be considered a separate cell, the vegetative cell). The vegetative cell forms the pollen tube, and a further mitotic division of the generative nucleus forms two sperm cells, each with its own plasma membrane and limited wall. The latter division can occur before or after germination so that each pollen grain is either released at the two-celled stage (one vegetative cell and one generative cell) or at the three-celled stage (one vegetative cell and two sperm cells). In most angiosperms, pollen grains are shed at the two-celled stage (Shukla et al. 1998). When the pollen grain germinates, the pollen tube will grow out from an aperture point at an extremely rapid rate (Bedinger 1992; Taylor and Helper 1997) and if only a single generative cell is present, it will divide in the tube (Bedinger 1992; Taylor and Helper 1997; Shukla et al. 1998). The stage at which the pollen grains of *Arceuthobium* are shed is not clear.

Pollen grains develop complex walls throughout meiosis and mitosis. The highly resistant outer wall of the pollen grain, the exine, forms around haploid cells, but includes material derived from the tapetum (as mentioned). The exine of mature pollen grains is composed largely of an electron dense material called sporopollenin; its presence is necessary for most species to produce a viable pollen tube, as it is remarkably resistant to degradation (Bedinger 1992; Taylor and Helper 1997). The inner wall of the pollen grain is the intine. In the angiosperm *Nelumbo*, the intine forms rapidly during the earliest mature pollen stage and the mature pollen grain has three apertures, the points where the pollen wall has thinned (Kreunen and Osborn 1999). Hawksworth and Wiens (1972) defined the approximate average size

of *Arceuthobium's* pollen grain as 23 μm in diameter; however, the details concerning the development of the pollen grain remain unclear.

Purpose

The objectives of this study are to examine the developmental processes that take place during anther development, to describe the eventual three-dimensional relationship between the columella and whole anther, and to examine the mature pollen in the dwarf mistletoe species, *A. americanum* using modern light and dissecting microscopy techniques.

MATERIALS AND METHODS

Site Description and Sample Collection

Staminate *Arceuthobium americanum* plants with floral buds were collected by Kathryn Pernitsky from March 2010 and to April 2011. Sampling was done in an area adjacent to Stake Lake, 30 km south of Kamloops, BC (50° 31′ latitude and 120° 28′ longitude). Thus, this site was chosen because of its high degree of *A. americanum* infection. The scheduling of sample collection ranged from twice per week to once every two weeks, depending on the likelihood of major developmental events occurring (according to guidance by Ross Friedman, pers. comm.). Samples were collected from the same haphazardly- selected trees (marked with flagging tape and numbered) over the sampling period in order to reduce the extent of genotypic variation, and all samples collected were taken from eye level. From each tree a minimum of ten male samples (aerial shoots) were removed each time, with the average number of flowers per shoot around twenty-one. Samples were stored in Karnovsky's fixative (Karnovsky 1965) at the site and moved to a 4°C refrigerator once in the lab. These samples were stored for approximately two years in 10 mL glass vials sealed with plastic caps and made available for developmental analysis in the spring of 2012. From the available sample dates approximately twenty-one were used with the majority being from July, August, and September; although samples from February, March, April, and June were used as well (Table 1). Data were from *A. americanum* samples from four different trees.

Table 1. Sampling months, number of sample dates per month, and the number of trees that were sampled for *A. americanum* for each sampling date.

Month	Number of sample dates	*Samples available from more than one tree?
February	1	Not indicated
March	4	Yes, all four trees
April	2	Not indicated
June	2	Not indicated
July	4	Some (2 of 4) dates indicate all four trees were sampled
August	5	Some (2 of 5) dates indicate all four trees were sampled
September	3	Not indicated, but more than one glass vial with samples per date

*Note: for each date that included samples from more than one tree (or more than one glass vial per date), five (minimum) floral buds were dissected away from the main stem of each plant per date per tree (vial) and added to a single glass vial for infiltration. Of those, ten randomly selected buds were embedded in the micromould for sectioning.

Dissection Microscopy

Samples were chosen for dissection based on the state of the flower. That is, only partially opened flowers with closed anthers and plants with large buds (presumably about to open, i.e., approaching anthesis) were dissected. Under the fume hood, Karnovsky's fixative was removed from glass vials containing whole samples (entire aerial shoots with floral buds and stems), and replaced with deionized, distilled water (DDW). On the bench, the whole samples were washed with DDW three times with a minimum of 15 minutes between each change. On average, five whole samples per date were viewed and dissected using a

combination of razor blades, scalpels, and forceps under a Nikon SMZ 1500 dissecting microscope. The dissecting microscope was equipped with a Nikon Coolpix 995 camera for photographing the samples and a Toshiba television for live viewing. Digital images were modified with Windows Live Photo Gallery. Drawings of dissected samples were also made from this microscope.

Light Microscopy

All steps were performed at room temperature unless otherwise noted.

Processing - Dehydration

In a fume hood, fixed samples of individual staminate flower buds were dissected from the stem using forceps, razor blades, and scalpels in various combinations with the aid of a Kyowa Optical (model SDZ-PL) dissecting microscope and placed back into the Karnovsky's fixative prior to embedding, approximately an hour. Between five and ten floral buds were dissected from each sampling date provided and if more than one tree was sampled per date, at least five floral buds per tree were dissected away from the main stem. In the fume hood, the dissected samples were washed a minimum of three times with deionized, distilled water for a minimum of 15 minutes each. Following the three DDW washes, the samples were moved to the bench and serially dehydrated via ethanol washes. All samples were immersed in 30%, 50%, 70%, and 90% ethanol for a minimum of 20 minutes each, as well as three changes of 100% denatured ethanol (90% anhydrous ethyl alcohol, 5% methyl alcohol, 5% isopropyl alcohol).

Processing – Infiltration and Embedding

Infiltration began by replacing the discarded 100% ethanol from the final wash with a 1:1 solution of LR White Resin and 100% ethanol. The 1:1 solution was allowed to infiltrate the samples for a minimum of 12 hours on an Ames Aliquot Mixer, although longer infiltration times (24 hours) were most successful. Next, the same infiltration procedure was followed with a 3:1 solution of LR White and 100% ethanol. Finally, the samples were infiltrated with three changes of 100% LR White, each on the rotator for at least 12 hours - although allowing the samples to remain in the resin for three days between each change was optimal. To polymerize and embed, each sample was extracted from the 100% LR White and positioned at the base of a 10 capsule micromould with the samples orientated so that the tip of the bud was either facing down (oriented for cross sectioning) or on its side (oriented for longitudinal sectioning). Each micromould was filled to the top with a mixture of LR White and LR White Accelerator (10 mL: 1 drop, respectively) and allowed to harden for minimum one hour. Samples tended to reorient with the addition of the polymerization mixture to the micromould and in order to counter the change of orientation, a small wooden probe was used to reorientate any notably skewed samples.

Sectioning

In preparation for sectioning, each block with an embedded sample was removed from each micromould with a razor blade. Using a razorblade, the blocks were then trimmed to a trapezoidal blockface under a dissecting microscope prior to sectioning with a microtome. Glass knives were made using a LKB Bromma 7800 Knifemaker, equipped with photographic tape water troughs ("boats"), and sealed with wax.

The glass knives were used with a Sorvall MT2-B Ultra microtome to make thin sections (about 3-5 μm in thickness) that were removed from the boats with a flattened wooden probe and floated on single drops of DDW on Superfrost Plus Gold Slides. In a fume hood, the slides were placed onto a GCA/Precision Scientific slide warmer set to 55°C and covered with a petri dish lined with cloth and moistened with xylenes (to aid the flattening/dewrinkling of each section).

Staining and Viewing

Sections were stained with crystal violet for observation using light microscopy. The crystal violet solution was comprised of 10 g crystal violet dissolved in 100 mL 95% ethanol and 4 g ammonium oxalate dissolved in 400 mL of DDW. The crystal violet (10 g/0.5 L) stain was added to the slides with a syringe equipped with a 0.45 μm nylon filter (about one drop per section) and allowed to sit for thirty seconds to one minute, after which they were rinsed with DDW. Rinsed slides were then placed back on the slide warmer to dry.

An Olympus CX2ILED compound light microscope was used to survey the stained sections allowing the creation of preliminary drawings. On average, approximately forty sections per sample were made with a minimum of five samples per sample date, totaling approximately 200 sections per sample date (minimum). A Nikon Eclipse E400 microscope equipped with a Nikon Coolpix P6000 camera was used to photograph selected sections. Digital images were modified with Windows Live Photo Gallery.

RESULTS

Anther Development

The perianth (non-reproductive segments) of the male flower of *A. americanum* is typically comprised of three sepals, and no petals, with each sepal bearing a stalk-less (sessile) anther (Figure 1). At the center of the flower is a tri-lobed central cushion of tissue whose lobes alternate with the perianth segments. Shoot tips of the developing inflorescences have a single terminal bud with two inferior lateral buds arranged in opposite orientation (Figure 2). Taller stems likely have lateral buds below those inferior to the terminal bud, which are decussate (perpendicular) to the first set of paired lateral buds. Most male flowers of *A .americanum* have three perianth segments (i.e., are trimerous); however, some terminal and lateral buds observed had four perianth structures (and four anthers).

Serial cross-sections of immature floral buds (oblique sections of the anthers) revealed unilocular anthers encased by three cell wall layers (Figure 3). The outer most layer is the epidermis, the middle layer is the sub-epidermal layer, and the innermost layer is the tapetum. These layers are generally all uniseriate. There is a point at the base of the anther's connection to the perianth where the epidermal layer becomes two or three cells thick (Figure 4). Also, the epidermal layer develops thickenings in the periphery of the epidermal cells near to the time of dehiscence (Figure 5).

Serial sections of both developing and mature floral buds, along with mature anther dissections, revealed that the anther of *A. americanum* is penetrated by a central column of sterile tissue or columella (Figures 6, 7, 8, and 9). In many of the developing floral bud cross sections, the anthers appeared to be oddly penetrated by

the columella (Figure 6). The extent to which the columella seemed to penetrate the anther depended upon the minor degrees of rotation that the samples may have undergone during polymerization (block formation). Longitudinal sections of developing floral buds revealed a similar pattern of penetration into the anther by the columella, although in these sections, the penetration occurred in the lateral plane (Figure 7). In cases where serial cross sectioning of the developing and mature anthers were achieved, these showed that the columella did not seem to span the transverse axes of the anther in either direction; in addition, the columella did not seem to directly contact the apex of the loculus in the vertical axis (opposite to where the anther connects to the perianth at the base of the anther). Instead, a few cells, likely tapetal cells, bridged the gap between the columella and the apex of the loculus.

In the latter phases of development, the epidermal layer contains vacuoles, and although crushed, the middle layer has visible, prominent nuclei (Figure 10). The tapetal layer does not appear to have begun programmed cell death and is also uninucleate. Tapetal cells in the later stages of development appeared irregular in shape. Just prior to and during tapetal degeneration, light-staining extraneous material, likely of tapetal origin, appears in the loculus. The darker staining, thicker "spots" in the loculus are the spiny exines of pollen grains (as seen by serial sectioning). Upon closer examination of the microspore cell walls, a thickened intine below each of the three apertures as well as a thinned, spiny exine can be observed (Figure 11). The intine and the exine appear to remain uniform in thickness at all other locations on the pollen grain surface

Mature Anther

Dissection of the mature anther confirmed that the columella does not span the horizontal length of the anther (Figure 8); however, the columella structure did seem to run the length of the vertical axis (although examination of the serial sections suggested cells of a different type may be bridging a small gap) to such an extent that suggests a connection between the anther wall and the columella (represented in Figure 9). The connection likely causes a central groove in the anther's outer surface.

The mature anther wall consists of only one layer of functional cells - the epidermal layer, which has developed thickenings and is, therefore, more appropriately named the exothecium (Figure 12). At this time (anther maturity), mature pollen grains are present in the anther's loculus, the tapetum has completely degenerated, and the remnants of the middle layer appear as a thin covering on the inner side of the epidermal (exothecial) layer (Figure 12). The mature pollen grains contain two nuclei, the generative nucleus and the vegetative nucleus. No obvious plasma membrane or wall circumscribed the generative nucleus, but such a tenuous boundary might not be detectable by light microscopy. The generative nucleus is round in shape and the vegetative nucleus is fairly elongate. Both nuclei are central to the pollen grain and in relatively close proximity to one another. It is at this stage that the pollen will be shed.

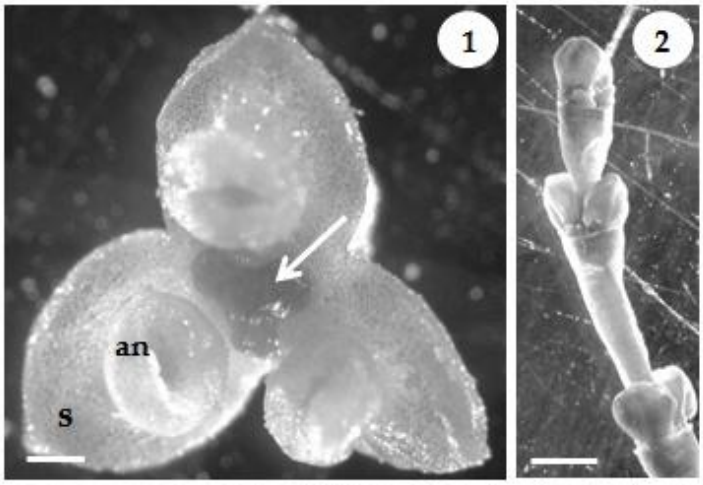
Figure 1. Dissecting micrograph of a fixed male flower. An open trimerous flower with three sepals (s), each with a sessile anther (an) surrounding the tri-lobed central cushion (arrow). Scale bar 0.5 mm.

Figure 2. Dissecting micrograph of a fixed inflorescence with a single terminal floral bud and paired lower lateral buds. Scale bar 2 mm.

Figure 3. Light micrograph of a serial sectioned (CS) anther revealed a unilocular anther. Scale bar 85 μm.

Figure 4. Light micrograph of an anther (LS). The connection between the base of the anther and the perianth is two or three epidermal cells thick (arrows). Scale bar 23 μm.

Figure 5. Light micrograph of epidermal cells near to the time of dehiscence. Epidermal thickenings are visible at the cell periphery (arrows). Scale bar 25 μm.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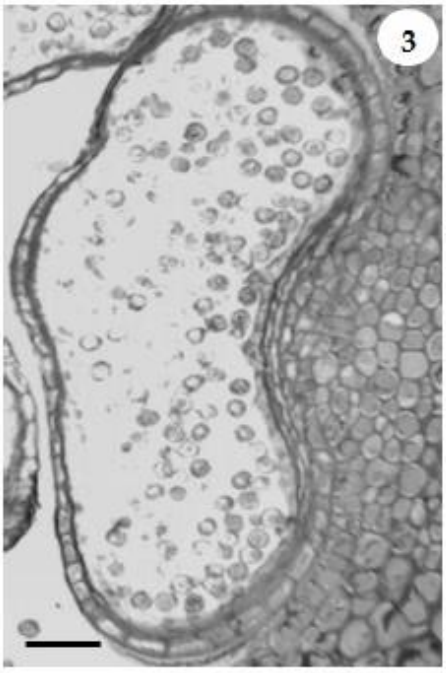
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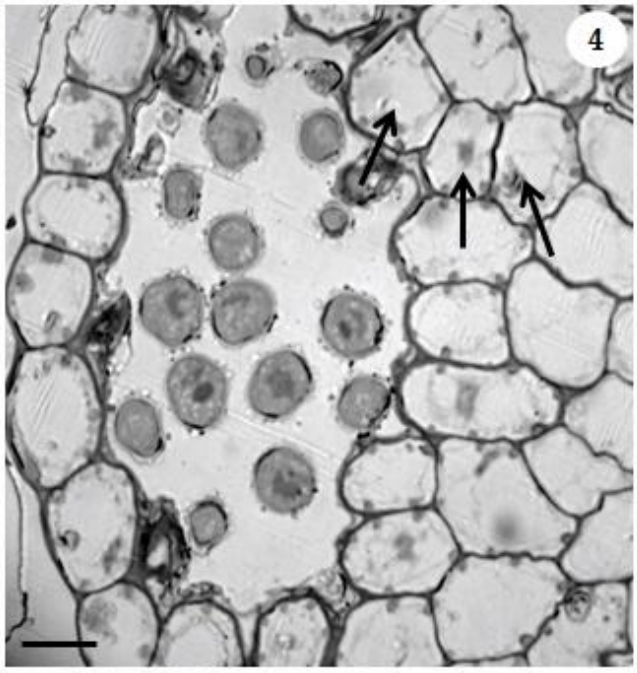
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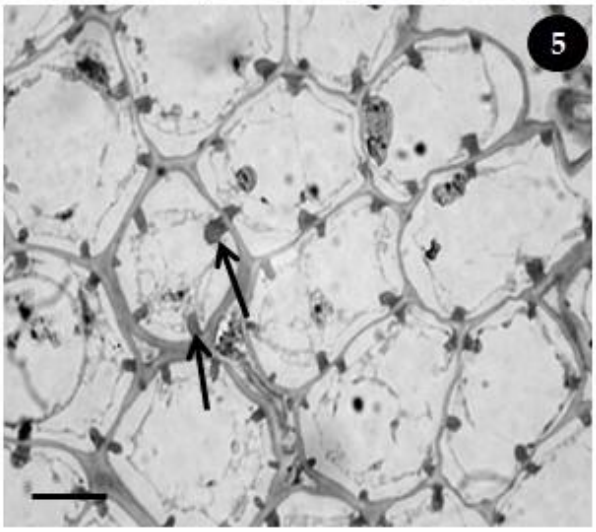
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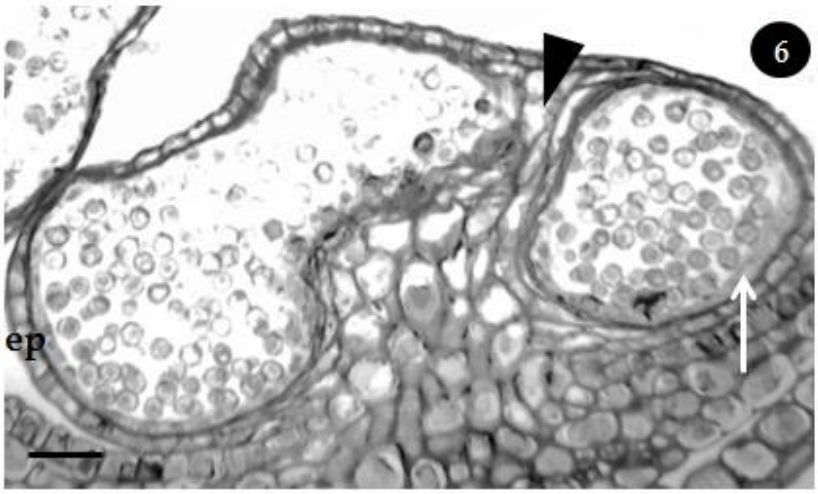
Figure 6. Light micrograph (CS) of an anther with columella (arrow head) tissue partially dividing the loculus. Also, evident are the tapetum (white arrow) and the epidermis (ep). Scale bar 82 μm.

Figure 7. Light micrograph (LS) of an anther with a partially penetrating columella (arrow head) and a fully connecting columella (arrow). Scale bar 160 μm.

Figure 8. Dissecting micrograph of a dehisced anther with fully developed pollen grains (pg) and the central columella (arrow). Scale bar 0.4 mm.

Figure 9. Diagrammatic representation of the eventual relationship between the columella (c) and the anther (longitudinal section). The columella is partially dividing the loculus with developing pollen grains (pg).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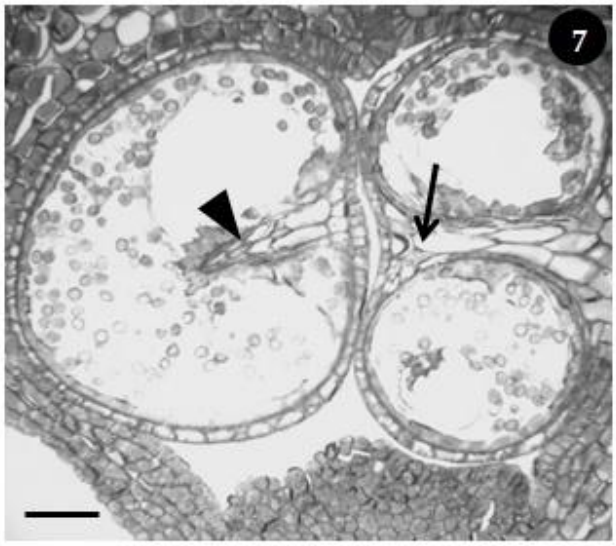
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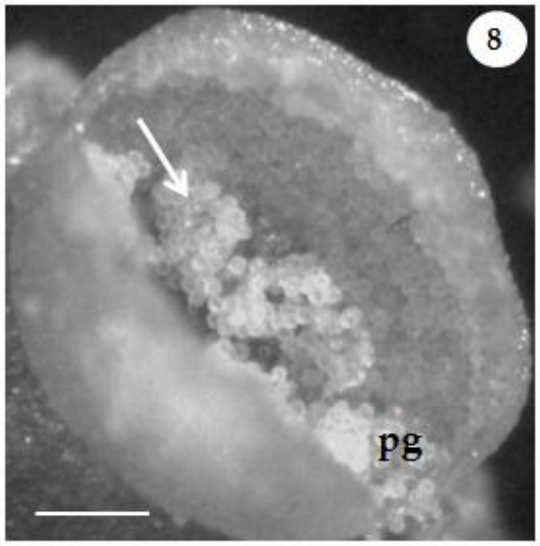
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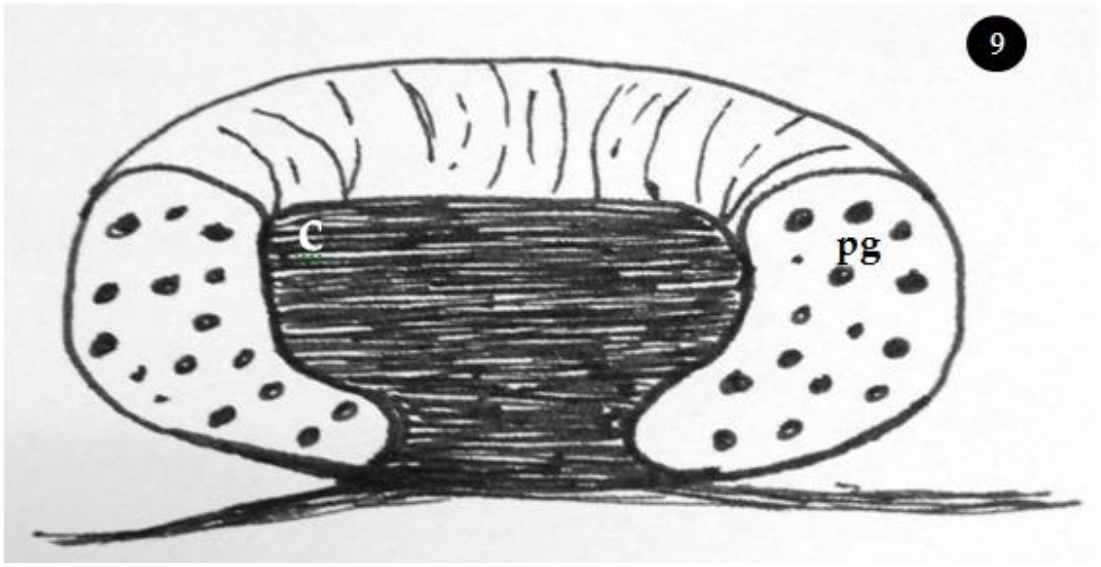
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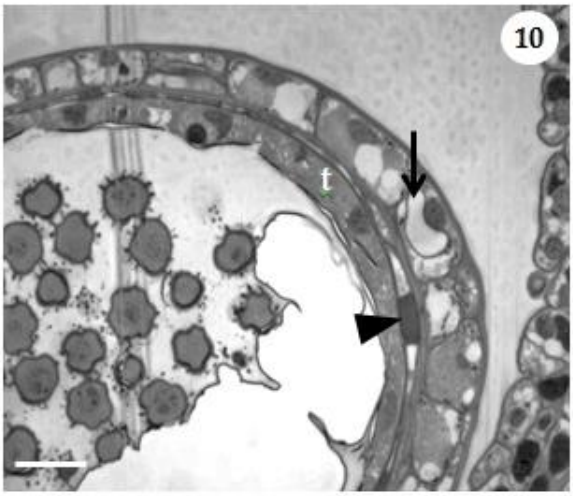
Figure 10. Light micrograph (CS) of maturing pollen grains in an anther with a vacuolate epidermis (arrow), uninucleate and crushed middle layer (arrow head), and an intact, uninucleate tapetal layer (t). Scale bar 40 μm.

Figure 11. Light micrograph of young pollen grains (CS). The pollen aperture points display a thinning exine and a thicker intine (arrows). The tapetum (t) has begun programmed cell death and some lightly staining material (likely) of tapetal origin can be seen in the loculus (arrow head). Scale bar 15 μm.

Figure 12. Light micrograph of and anther (LS) with mature pollen grains. Both the generative (gn) and vegetative nuclei (vn) are centrally located in the pollen grain. The only remaining anther wall layer prior to dehiscence is the epidermis (ep). Scale bar 18 μm.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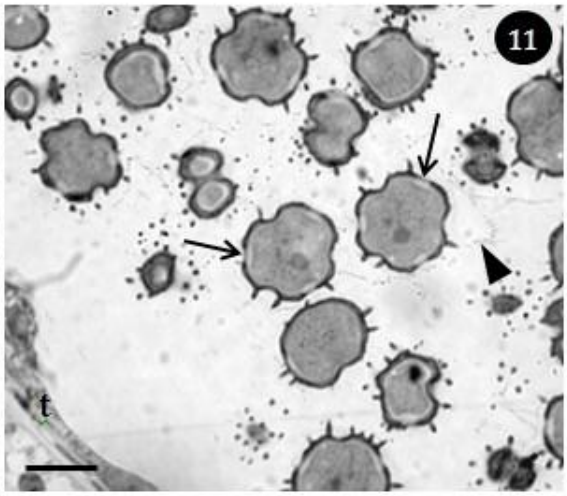
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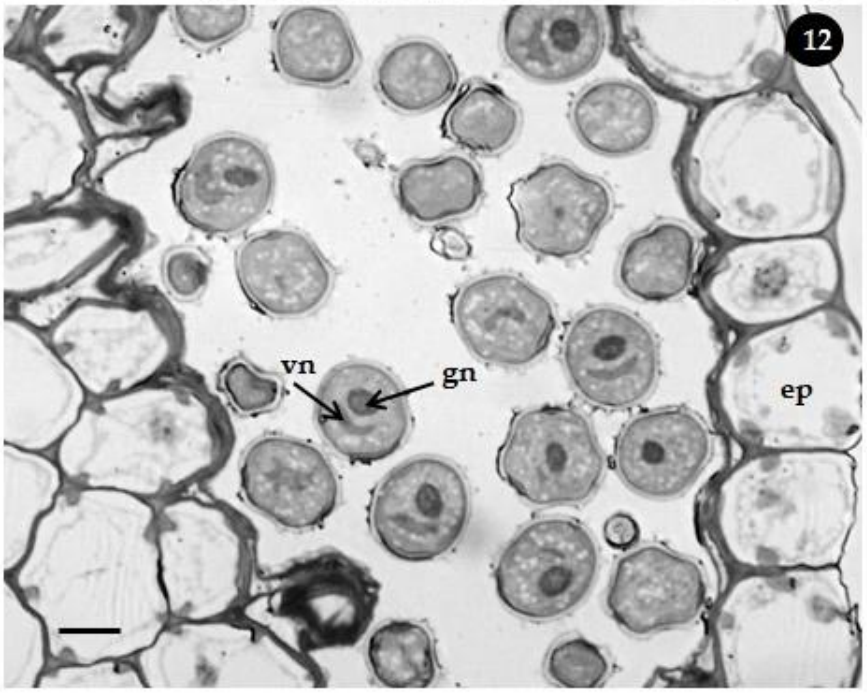
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DISCUSSION

Anther Wall Development

The characteristic of the middle layer's eventual crushed state and the tapetal layer's degeneration in *A. americanum* is not unique to the species; many angiosperms anther walls undergo a similar process. Davis (1966) noted that the middle layers of angiosperms lack the ability to divide anticlinally. This disability handicaps the middle layer's ability to adjust to the increasing size of the microsporangium as the microsporogenous tissue divides within.

Typically, the tapetal layer of angiosperms is composed of a single cell layer, as in *A. americanum*; however, in some species, the tapetum is comprised of two cell layers resulting from periclinal divisions (Davis 1966). Serial sectioning of the *A. americanum* anther revealed a uninucleate tapetum, an observation that is in agreement with Cohen (1968), although other genera closely related to *Arceuthobium* within the family Santalaceae (ascribed to the former family Viscaceae) have bi- or tetranucleate tapetal cells (Davis 1966). Throughout development, but more prominently seen in the later stages of development, the tapetal cells of *A. americanum* exhibited odd cellular organization and some irregularities in cellular shape. That is, the cells were not defined like those of the epidermis; rather, they appeared more elongate and fluid, like sickle-cell erythrocytes, sometimes overlapping just at the edges. This observation seemed independent from the plane of sectioning. Tapetal disorganization seems to occur as the result of orbicule (small sporopollenin bodies associated with secretory tapeta) detachment, secretion, and support substance secretion (Shukla et al. 1998; Furness and Rudall 2001). Also, the tapetal cells remain associated with the anther wall until their degeneration;

therefore, the tapetum of *A. americanum* seems to exhibit the characteristics of a secretory tapetum.

Davis (1966) stated that members of the family Santalaceae follow a dicotyledonous type of anther wall ontogeny, whereas the former family Viscaceae fits the monocotyledonous type of anther wall development (Figure 3i). However, with the absence of an endothecium neither, neither of these two descriptions of anther development completely fits *Arceuthobium*. Cohen (1968) describes the developing microsporangium as a modified form of dicotyledonous or reduced type. I believe that the pattern of *A. americanum's* anther wall ontogeny is better ascribed to a modified dicotyledonous type, as Davis (1966) definitively stated that the reduced type does not form a middle layer. However, an argument can be made for ascribing *A. americanum* to a modified form of the reduced type of anther wall ontogeny, as *A. americanum* does not seem to develop a secondary parietal layer, a condition for the dicotyledonous type. Therefore, it may be necessary to create a new category (type) of anther wall development to best describe *Arceuthobium*.

Mature Anther Wall

An unusual aspect of *A. americanum's* anther wall is the development of thickenings in the epidermal layer along with the absence of an endothecium. Normally, the endothecial layer is present directly below the epidermis and functions in normal, non-explosive dehiscence (Maheshwari 1950). Other angiosperms that lack an endothecium, but have epidermal thickenings at maturity (that is, have an exothecium) are those belonging to the family Ericaceae, specifically from the genera *Kalmia* and *Phyllodoce* (Skene 1948; Davis 1966; Kron et al. 2002). The development of thickenings in the epidermal cells of the microsporangium is also present in all

gymnosperms, except *Ginkgo* (Bhatnagar and Moitra 1996). Interestingly, *Ginkgo* develops its thickenings in a sub-epidermal layer, similar to the typical angiosperm.

The epidermal layer of the sporangia in Leptosporangiate ferns has a specialized spine of thickened cells (the annulus) intermixed with vacuolated cells filled with water, comprising what is known as a "plant catapult" (Noblin et al. 2012). When the cells between the annulus lose water to the environment, there is a decrease in the cell volume. As a result, the tension created is focused on the aperture point and the sporangia open very rapidly. At this stage, many of the spores remain in the head of the plant catapult, and it is not until the head springs back that the spores are sent flying. Because of some structural similarities between the fern sporangia and the epidermal cells of the *A. americanum* anther (that is, vacuolated cells with thickenings), there may be some mechanistic cross-over. Perhaps as the moisture held in epidermal cells of *A. americanum* evaporate, the cell volume would decrease and tension would build on the connection between the anther wall and columella. Finally, when the tension becomes greater, the anther wall would burst open along a central groove (transverse slit) that corresponds to the columella's position beneath. When the anther opens in this manner, some pollen would be sent into the air, although the majority would remain in the anther to be dispersed via the wind or by insects. Some angiosperms that do not develop an endothecium dehisce via the dissolution of certain cells in the anther wall, which could cause further weakening in the structural integrity of the anther wall; this could allow the anther burst open with more ease (Maheshwari 1950). However, I did not note any evidence of anther wall cellular dissolution in mature anthers with mature pollen grains.

Columella

A unique aspect of *A. americanum's* anther is the sterile tissue called the columella, which penetrates the loculus of each anther. The columella has been the subject of much disagreement amongst researchers for nearly one hundred years, although there have not been any recent studies (within the last twenty to thirty years) that have attempted to characterize the nature of *Arceuthobium's* columella with modern microscopy. Previously, the columella of *A. americanum* was described as extending the total width of the anther, i.e., along one of the transverse axes, in either one direction or both directions (Dowding 1931) or as being a central column with a toroid of sporogenous tissue surrounding it (Dowding 1931; Cohen 1968). The confusion is understandable, as serial cross sections of the buds often generate oblique sections through the anther. In this study, such sections that just began to enter the anthers obliquely revealed unilocular anthers, each anther having a relatively uniform oval to kidney shape that seemingly lacked a columella. However, as the sectioning continued, columella tissue began to become apparent at the base of the anther, almost as though it penetrates the anther from the connection point of the anther to the perianth. Further sectioning into the anther revealed a connection between the anther and the outer epidermal tissue, seeming to partition the locule in two or give the anther a three-dimensional toroid-like structural design; both observations are actually incorrect as continued sectioning revealed, once again, a unilocular anther.

Longitudinal sectioning of the buds was also used in an attempt to further understand the overall three-dimensional arrangement. Due to the trimerous nature of the male flower, longitudinal sections usually only captured two anthers at a time, and were difficult to interpret, as entrance into each of the anthers was never

completely symmetrical. Therefore, typical longitudinal sections would consist of two anthers, one with a fairly symmetrical circular shape lacking columella tissue, and another oval shaped anther with the loculus partitioned in two by columella tissue. This sectional morphology is explained by partial entrance into one anther (the circular anther), i.e., not deep enough into the anther to see evidence of the columella. The anther being partitioned in two appears as such due to the depth of the sectioning, i.e., in deep enough to note the presence of the columella partitioning the anther.

After dissecting numerous anthers, and in conjunction with the light microscopy data, it was concluded that the columella does not span the width (transverse axes) of an *A. americanum* anther at maturity. Dowding (1931) claimed to see a considerable amount of variation with regard to columella orientation in the mature unilocular anther (although the columella positions she observed were only ever parallel to the transverse plane or at right angles to the transverse plane), but in this study, all dissected samples were relatively homogeneous (parallel to the transverse plane). However, I do believe that Dowding (1931) only examined serial sections and did not include whole dissection in her study; therefore, the variation she observed could have been due to the two dimensional nature of her work. Even in this study, I found it challenging to make three dimensional conclusions from my two dimensional sections alone.

The conclusions drawn by Dowding (1931) and Cohen (1968) are not dissimilar to what can be seen during serial sectioning. However, when serial sectioning of varying orientations is completed in combination with whole specimen dissection, as performed here, the description becomes clear; the eventual three-dimensional shape of the mature anther can be described as being penetrated, from the base of

the anther, by the columella, which extends up the vertical axis, near to the total height of the anther (likely making a full connection via an extended tapetum) and only extends about half the transverse width of the anther in one direction. The anther wall attaches to the columella, which results in a central groove in the outer anther surface, contributing to a cushion-like appearance. Dowding (1931) noted that the connections between the anther wall and columella were of tapetal origin. Because of this connection, the columella may function in pollen dehiscence or as a means of maintaining the structural integrity of the mature anther such that the anther wall does not cave in at maturity.

The discrepancies between my observations and those of Dowding (1931) could have been due to the age of the samples being dissected, as Dowding (1931) looked at all ages, whereas in this study, dissected samples were in the latter stages of the anthers' development; therefore, it is possible that certain aspects of the columella abort such that the eventual anther is quite different from the early anther. Mauseth (1988) argues that the *Arceuthobium* loculus is initially bisporangiate or even tetrasporangiate (and becomes unilocular at dehiscence due to the breakdown of the dividing septum). However, I have only observed unilocular anthers in agreement with Dowding (1931), Cohen (1968), and Takhtajan (2009).

The cellular origin of the columella, then, because a question worthy of future pursuit. It is likely that some early repeated periclinal divisions of the primary parietal layer reach into the loculus, forming the columella. Or perhaps, since the tapetum may form from connective tissues (Davis 1966; Anjaneyulu and Lakshminarayana 1989), the same is true for the columella, which does seem to connect to the tapetum. More work is needed.

Whole specimens collected on April 16th had partially opened flowers (but fully closed anthers), and upon dissection, the anthers burst open. The samples were likely dehydrating as a result of the heat from the microscope lights; therefore, the columella and the anther wall would have been simultaneously drying out, creating more and more tension until finally the anther burst open sending a small amount of pollen up into the air. Prior to the start of dehiscence, the anther of *Ricinus communis* consists of a central septum attached to the stomium, i.e., the point at which rapture occurs in a pollen sac to release the pollen (Bianchini and Pacini, 1996); this arrangement is similar to the pre-dehiscence anther of *A. americanum*. The opening of the *R. communis* anther begins with the separation of the stomium from the septum. A similar process may also occur in *A. americanum*, with the columella being considered homologous to the septum. From this stage, the anther of *R. communis* opens as the stomium cells separate, but the pollen is not yet released. Rather, the loculus walls first separate and turn inside-out, completely exposing the pollen, which remains anchored via the pollenkit (adhesive material that surrounds pollen grains). The pollen is then explosively ejected from the anther when the loculus rapidly returns to its original position. The anther of *A. americanum* does not seem to rapidly return to its starting position, to disperse pollen; however, it can close in response to low temperatures and increased humidity, to protect and conserve the pollen (Gilbert and Punter, 1990). This unique method of controlling pollen dispersal restricts the exposure of *A. americanum* pollen to conditions that favor dispersal such as warm, dry, windy days.

Mature Pollen

The mature pollen of *A. americanum* has three aperture points, two nuclei (a generative and vegetative nucleus), and a spiny exine. No obvious newly-formed

cellular boundary surrounded the generative nucleus, but such a boundary comprised of a plasma membrane and perhaps some cellulosic wall material is likely present; transmission electron microscopy would probably reveal that the generative nucleus is circumscribed in its own cell. Because the generative nucleus does not seem to undergo a second mitotic division before the pollen is shed, each mature pollen grain is probably released at the two-celled stage. This characteristic is similar to that of most angiosperms (Shukla et al. 1998).

Conclusion

There are notable deviations regarding the anther of *A. americanum* when compared with typical angiosperms: the endothecium never develops; the epidermis develops fibrous thickenings, called the exothecium; and a central column of sterile tissue remains present in the mature anther, called the columella. These deviations might be explained by an overall evolutionary reduction in *Arceuthobium*. Similar to most other angiosperms, *A. americanum's* tapetum is likely the secretory type and pollen grains are probably shed at the two-celled stage. This work contributes to the growing body of information contributing to the understanding of *A. americanum*, the genus *Arceuthobium*, and angiosperms as a whole.

LITERATURE CITED

Angiosperm Phylogeny Group. (2003). An update of the Angiosperm Phylogeny Group classification for the orders and families of flowering plants: APG II. Botanical Journal of the Linnean Society **141:** 399-463.

Anjaneyulu, C., and Lakshminarayana, K. 1989. Microsporogenesis and the development of male gametophyte in two species of *Diospyros* L. Taiwania, **34**: 187-91.

Bedinger, P. 1992. The Remarkable Biology of Pollen. The Plant Cell **4:** 879-887.

Bhatnagar, S.P., and Moitra, A. 1996. Gymnosperms. Daryaganj, New Delhi: New Age International (P) Limited, Publishers.

Bianchini, M., and Pacini, E. 1996. Explosive Anther Dehiscence in *Ricinus communis* L. Involves Cell Wall Modifications and Relative Humidity. International Journal of Plant Sciences **157:** 739-45.

Calvin, C.L., and Wilson, C.A. 1996. Endophytic system. *In* Dwarf Mistletoes: Biology, Pathology, and Systematics. *Edited by* F.G. Hawksworth and D. Wiens. Agriculture Handbook 709, USDA Forest Service, Washington, D.C. 113-122.

Chhikara, A., and Ross Friedman, C.M. 2008. The effects of male and female *Arceuthobium americanum* (lodgepole pine dwarf mistletoe) infection on the relative positioning of vascular bundles, starch distribution, and starch content in *Pinus contorta* var. *latifolia* (lodgepole pine) needles. Botany **86:** 539–543.

Clément, C., Laporte, P., Audran, J.C. 1998. The loculus content and tapetum during pollen development in *Lilium*. Sexual Plant Reproduction **11:** 94–106.

Cohen, L.I. 1968. Development of the staminate flower in the Dwarf Mistletoes, *Arceuthobium*. American Journal of Botany **55:** 187-193.

Davis, G.L. 1966. Systematic Embryology of the Angiosperms. New York: John Wiley & Sons.

Dowding, E.S. 1931. Floral morphology of *Arceuthobium americanum*. Botanical Gazette **91:** 42-54.

Drummond, D.B. 1982. Timber loss estimates for the coniferous forests of the United States due to dwarf mistletoes. USDA Forest Service, Fort Collins, Colorado. Rep. 83-2.

Dundas, I.S., Saxena, K.B., Byth, D.E. 1982. Pollen mother cell and anther wall development in a photoperiod-insensitive male-sterile mutant of pigeon pea (*Cajanus cajan* (L.) Millsp.). Euphytica **31:**371–375.

Furness, C.A., and Rudall, P.J. 2001. The tapetum in basal angiosperms: early diversity. International Journal of Plant Sciences **162:** 375-392.

Gilbert, J., and Punter, D. 1990. Release and dispersal of pollen from dwarf mistletoe on jack pine in Manitoba in relation to microclimate. Canadian Journal of Forest Research **20:** 267-73.

Greyson, R.I. 1994. The Development of Flowers. New York: Oxford University Press.

Goldberg, R.B., Beals, T.P., and Sanders, P.M. 1993. Anther development: basic principles and practical applications. Plant Cell **5:** 1217-1229.

Hardy, C.R., Stevenson, D.W., and Kiss, H.G. 2000. Development of gametophytes, flower, and floral vasculature in *Dichorisandra thyrsiflora* (Commelinaceae). American Journal of Botany **87:** 1228-1239

Hawksworth, F.G. 1954. Observations on the age of Lodgepole Pine tissues susceptible to infection by *Arceuthobium americanum*. Phytopathology **44:** 522.

Hawksworth, F.G., and Wiens, D. 1972. Biology and Classification of Dwarf Mistletoes (*Arceuthobium*). Agriculture Handbook 401, USDA Forest Service, Washington, D.C.

Hawksworth, F.G., and Wiens, D. 1996. Dwarf Mistletoes: Biology, Pathology, and Systematics. United States Department of Agriculture Forest Service. Washington.

Jerome, C.A., and Ford, B.A. 2002. The discovery of three genetic races of the dwarf mistletoe *Arceuthobium americanum* (Viscaceae) provides insight into the evolution of parasitic angiosperms. Molecular Ecology **11:** 387-405.

Karnovsky, M.J. 1965. A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. Journal of Cell Biology **27:** 137A.

Kreunen, S.S., and Osborn, J.M. 1998. Pollen and anther development in *Nelumbo* (Nelumbonaceae). American Journal of Botany **86:** 1662-76.

Kron, K.A., Judd, W.S., Stevens, P.F., Crayn, D.M., Anderberg, A.A., Gadek, P.A., Quinn, C.J., and Luteyn, J.L. Phylogenetic Classification of Ericaceae: Molecular and Morphological Evidence. The Botanical Review **68:** 335-423.

Maheshwari, P. 1950. An introduction to the embryology of angiosperms. New York: McGraw-Hill.

Mauseth, J.D. 1988. Plant Anatomy. Menlo Park, California: The Benjamin/Cummings Publishing Company.

Noblin, X., Rojas, N.O., Westbrook, J., Llorens, C., Argentina, M., and Dumais, J. The Fern Sporangium: A Unique Catapult. Science **335:** 1322.

Nickrent, D.L., Garcia, M.A., Martin, M.P., and Mathiasen, R.L. 2004. A phylogeny of all species of *Arceuthobium* (Viscaceae) using nuclear and chloroplast DNA sequences. American Journal of Botany **91:** 125-38.

Pacini, E., Franchi, G.G., and Hesse M. 1985. The tapetum: Its form, function, and possible phylogeny in Embryophyta. Plant Systems and Evolution **149:** 155–185.

Pacini, E. 1990. Tapetum and microspore function. *In:* S. Blackmore and R.B. Knox (eds.), Microspores: Evolution and Ontogeny, pp. 213-237. London: Academic Press.

Pacini, E., and Franchi, G.G. 1991. Diversification and evolution of the tapetum. *In*: S. Blackmore and S. Barnes (eds.), Pollen and Spores: Patterns of Diversification, pp. 181-86. Verlag, Berlin: Springer.

Penfield, F.B., Stevens, R.E., and Hawksworth, F.G. 1976. Pollination ecology of three Rocky Mountain dwarf mistletoes. Forest Science **22:** 473-84.

Piffanelli, P., Ross, J.H.E., and Murphy D.J. 1998. Biogenesis and function of the lipidic structures of pollen grains. Sexual Plant Reproduction **11:** 65–80.

Scott, R.J., Spleman, M., and Dickinson, H.G. 2004. Stamen structure and function. Plant Cell **16:** S40-S60.

Shaw, C.G., III, and Loopstra, E.M. 1991. Development of dwarf mistletoe infections on inoculated western hemlock trees in southeast Alaska. Northwest Science **65:** 48-52.

Shukla, A.K., Vijayaraghavan, M.R., and Chaudhry, B. 1998. Biology of Pollen, (1st edition). Darya Ganj, New Delhi: APH Publishing Corporation.

Skene, M. 1948. Biology of Flowering Plants. Darya Ganj, New Delhi: Discovery Publishing House.

Smith, R.B. 1971. Development of dwarf mistletoe (*Arceuthobium*) infections on western hemlock, shore pine, and western larch. Canadian Journal of Forest Research **1:** 35-42.

Takhtajan, A. 2009. Flowering Plants, (2nd edition). Netherlands: Springer.

Taylor L.P., and Hepler P.K. 1997. Pollen Germination and Tube Growth. Annual Review of Plant Physiology & Plant Molecular Biology **48:** 461.

Thoday, D., and Johnson, E.T. 1930. On *Arceuthobium pusillum*, Peck. II. Flowers and fruit. Annals of Botany **44:** 813-24.

Tobe, H. 1989. The embryology of angiosperms: Its broad application to the systematic and evolutionary study. The Botanical Magazine of Tokyo **102:** 351–367.

Vasil, I.K. 1967. Physiology and Cytology of Anther Development. Biological Reviews **42:** 327–366.

Zhu, J., Lou, Y., Xu, X., and Yang, Z-N. 2011. A Genetic Pathway for Tapetum Development and Function in Arabidopsis. Journal of Integrative Plant Biology **53:** 892–900.